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EFFECTS OF STROMA FREE HEMOGLOBIN ON BLOOD PRESSURE AND RENAL FUNCTION IN THE HYPOTENSIVE RAT: POTENTIAL ROLE OF NITRIC OXIDE INACTIVATION BY HEMOGLOBIN

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The short term systemic and renal hemodynamic effects of two stroma free hemoglobin (SFH) solutions, one unmodified and the other modified by cross-linking, were examined in anesthetized rats following hemorrhagic hypotension. Both forms of SFH increased MAP and GFR to baseline, pre-hemorrhage values. The increase in MAP induced by unmodified SFH (from 61±6 to 111±7 mm Hg) was greater than the increase in MAP caused by an albumin solution iso-oncotic to the unmodified SFH solution (63±3 to

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82±2 mm Hg) ($p < 0.05$). Similarly, the increase in MAP associated with modified SFH infusion (56±4 to 96±5 mm Hg) was also substantially greater than that induced by an albumin solution of comparable oncotic pressure to the modified SFH (59±4 to 71±5 mm Hg) ($p < 0.05$). GFR increased in response to the unmodified SFH from 1.1±0.5 to 2.7±0.3 ml/min ($p < 0.05$), while the modified SFH inereased GFR from 0.7±0.2 to 2.7±0.3 ml/min ($p < 0.05$). As with MAP, the increase in GFR induced by both SFH solutions was greater than that associated with the oncotically matched albumin solutions.($p < 0.05$). In separate experiments, the effects of nitric oxide (NO) inhibition with L-NAME on MAP following hemorrhagic hypotension and subsequent infusion of unmodified SFH or albumin was also examined. In the albumin infused rats, L-NAME increased MAP from 82±4 to 104±7 mm Hg ($p < 0.05$). In marked contrast, NO inhibition with L-NAME had no further effect on MAP when infused after SFH (119±3 to 112±3, p = NS). We conclude that both unmodified and modified SFH solutions acutely improve MAP and GFR by the combined effects of intravascular volume expansion resulting from the colloid effect of the protein and by inactivation of NO.

ABSTRACT

The short term systemic and renal hemodynamic effects of two stroma free hemoglobin (SFH) solutions, one unmodified and the other modified by cross-linking, were examined in anesthetized rats following hemorrhagic hypotension. Both forms of SFH increased MAP and GFR to baseline, pre-hemorrhage values. The increase in MAP induced by unmodified SFH (from 61±6 to 111±7mmHg) was greater than the increase in MAP caused by an albumin solution iso-oncotic to the unmodifed SFH solution (63±3 to 82±2mmHg) ($p<0.05$). Similarly, the increase in MAP associated with modified SFH infusion (56±4 to 96±5mmHg) was also substantially greater than that induced by an albumin solution of comparable oncotic pressure to the modified SFH (59±4 to 71±5 mmHg) ($p<0.05$). GFR increased in response to the unmodified SFH from 1.1±0.5 to 2.7±0.3ml/min ($p<0.05$), while the modified SFH increased GFR from 0.7±0.2 to 2.7±0.3 ml/min ($p<0.05$). As with MAP, the increase in GFR induced by both SFH solutions was greater than that associated with the oncotically matched albumin solutions ($p<0.05$). In separate experiments, the effects of nitric oxide (NO) inhibition with L-NAME on MAP following hemorrhagic hypotention and subsequent infusion of unmodified SFH or albumin was also examined. In the albumin infused rats, L-NAME increased MAP from 82±4 to 104±7mmHg ($p<0.05$). In marked contrast, NO inhibition with L-NAME had no further effect on MAP when infused after SFH (119±3 to 112±3; p=NS). We conclude that both unmodified and modified SFH solutions acutely improve MAP and GFR by the combined effects of intravascular volume expansion resulting from the colloid effect of the protein and by inactivation of NO.

Key words: Blood substitutes; o-raffinose polyhemoglobin, hemorrhagic hypotension, nitric oxide.

INTRODUCTION

The availability of an acellular, stroma free hemoglobin (SFH) solution remains an important potential alternative to the use of blood transfusion (6). The original use of simple hemoglobin hemolysates were associated with a variety of serious side effects including kidney failure, intravascular coagulation, and anaphylactic reactions (1,24,25). However, it has become clear that removal of red cell stroma from the hemoglobin solutions reduces but does not prevent toxicity because the hemoglobin molecule itself has the potential to induce renal injury and dysfunction (3,9,24,25).

An understanding of the factors that mediate hemoglobin induced nephrotoxicity is important to facilitate the development of a non-toxic modified hemoglobin solution. It has been demonstrated in experimental models of intravascular hemolysis and rhabdomyolysis that the presence of hemoglobin and/or myoglobin in plasma can result in renal failure by a number of different mechanisms. The most widely recognized mechanism of hemoglobin induced renal failure is the nephronal obstruction that results from the intratubular precipitation of methemoglobin and free heme (26).

However, more recently, red cell-free plasma hemoglobin has been shown to have the potential to impair renal function in other ways. Hemoglobin can induce renal insufficiency by causing intrarenal vasoconstriction (12). These hemodynamic effects of cell-free hemoglobin have been demonstrated to be mediated, at least in part, by the inactivation of nitric oxide (NO) (10,13,22), a vasodilator constitutively produced by vascular endothelium which mediates vascular smooth muscle relaxation and plays an essential role in the regulation of blood presssure and renal function (16). Furthermore, recent experimental evidence has also emerged indicating that hemoglobin or its metabolites cause direct renal cell injury by accelerating the production of reactive oxygen species (7,17).

During the past 10-15 years a substantial effort has been made to develop modified SFH solutions that avoid toxicity to the kidney and other organs while retaining the ability of the molecule to serve as an effective oxygen carrier. Most attention has been paid to developing a modification that prevents the dissociation of the hemoglobin tetramer into dimers thereby minimizing the filtration of the hemoglobin at the glomerulus (6). These modifications, if effective, should serve to prolong the intravascular retention time and therefore increase the efficacy of the administered SFH. Furthermore preventing dimerization of the hemoglobin molecule should also minimize filtration of hemoglobin at the glomerulus and the renal toxicity associated with the intratubular precipitation of metabolites of hemoglobin such as methemoglobin and hematin (26).

The nephrotoxic effects of free intravascular hemoglobin have been found to occur predominantly in humans and animals that are volume depleted (9) . Since infusion of SFH is most likely to be necessary as a resuscitative fluid in volume depleted patients we have used a rat model of hemorrhagic hypotension developed in this laboratory (14) to compare the short term effects of an human hemoglobin which is either unmodified or modified by cross-linking with o-raffinose (o-raffinose polyhemoglobin (Hemosafe®)) (U.S. Patent No. 4,857,636 (1989)). In these experiments the concentrations of hemoglobin achieved in blood were insufficient to materially affect the oxygen carrying capacity of the blood.

METHODS

Male Sprague-Dawley rats (Charles River, Wilmington, MA), weighing between 250-350g were used for all experiments. Rats were fed regular Purina Rat Chow (Purina Mills, Chicago, IL) and allowed free access to water. Anesthesia was induced with an intraperitoneal injection of pentobarbitol sodium (5 mg/100 gm body wt) and then maintained with a constant intravenous

infusion of pentobarbitol (91 μg/min) throughout the study. Rats were placed on a thermostatically controlled heated table and body temperature was monitored with a rectal thermometer and maintained between 36 and 38°C. A tracheotomy was performed with the use of polyethylene (PE-240) tubing and the femoral artery was cannulated with PE-50 tubing for blood pressure monitoring as well as blood sampling. The left internal jugular vein was cannulated with two catheters of PE-50 tubing. A bladder catheter (PE-90) was placed by a suprapubic incision for urine sampling. GFR was measured by determining the clearance of methoxy-{^{3}H} inulin (New England Nuclear, Boston, MA). Inulin and pentobarbitol sodium dissolved in 5% dextrose water was infused into one venous catheter at a rate of 0.028 ml/min while the other internal jugular catheter was used for the infusion of vehicle (Ringers' lactate), albumin or stroma free hemoglobin.

Experimental Protocols

1. Comparison of the effects of modified and unmodified SFH and oncotically matched albumin solutions on MAP and GFR.

After an equilibration period of 30 minutes, two 20 minute control clearance periods were obtained during which blood pressure was measured continuously and urine collected for the measurement of inulin clearance, urine flow rate and sodium excretion. At the end of this period the rats were subjected to hemorrhage. Whole blood (20 ml/kg body wt) was removed through the femoral arterial catheter at the rate of 1ml/minute. After a further 45 minute equilibration period, a single 30 minute clearance ("post-hemorrhage" period), was obtained for the measurement of blood pressure inulin clearance, urine flow rate and sodium excretion.

Then, the rats were randomly divided into two experimental groups that received either the unmodified (n=8) or modified (n=7) SFH solution. Each experimental group was compared to two separate control groups that received

albumin solutions with oncotic pressures that matched the two SFH solutions (n=8 in each albumin group) (Table 1).

Following a further equilibration period of 15 minutes, two 20 minute ("post-infusion") clearance periods were obtained. At the end of the experiment, blood was rapidly obtained from the abdominal aorta for measurement of plasma hemoglobin.

2. Effect of nitric oxide synthase (NO) inhibition following SFH infusion.

In order to elucidate the role played by SFH induced NO inactivation in the hemodynamic effects of SFH we examined the effect of NO inhibition with Nω-nitro-L-arginine-methyl ester (L-NAME) following infusion of unmodified SFH in the hypotensive rat. The effect of L-NAME was also examined in two separate control groups; hypotensive animals infused with i) the vehicle (Ringer's lactate) or ii) an albumin solution iso-oncotic to the SFH. (Table 1)(n=4 in all groups).

After an equilibration period of 30 minutes, blood pressure was measured continuously for two 20 minute periods. At the end of this baseline period, the rats were subjected to hemorrhage as described above. After a further 45 minute equilibration period, MAP was measured for 30 minutes ("post-hemorrhage" period). Then, the rats were randomly divided into the three experimental groups that received either the vehicle (Ringer's lactate), albumin or unmodified SFH (n=4 in all groups). Following a further equilibration period of 15 minutes, MAP was measured during a 20 minute ("post-infusion") period. Then L-NAME (0.12mg/kg/min) was administered in all groups and MAP measured over two 20 minutes ("L-NAME period") following 15 minutes of equilibration.

Characteristics of the SFH solutions

Human hemoglobin was modified by crosslinking with raffinose-(0-raffinose polyhemoglobin, U.S. Patent No. 4,857,636 (1989)) provided by Hemosol Inc. (Etobicoke, Ontario, Canada). Unmodified SFH was human

hemoglobin that was purified by ultrafiltration without further modification. Both the albumin and SFH solutions were administered dissolved in 2ml of Ringers Lactate which was infused over 10 minutes. The concentrations and oncotic pressures of the two SFH solutions administered were different. The unmodified SFH was administered at a concentration of 17.3g/dL (oncotic pressure 92.4mmHg)(Table1). The concentration of the modified, cross-linked SFH was 11.7g/dL (oncotic pressure 21mmHg)(Table 1). Two albumin control solutions, oncotically matched to each SFH solution were infused in two separate control groups(Table 1).

The concentration of the unmodified hemoglobin preparation was higher than the modified SFH (Table 1). As a result, the rats receiving unmodified SFH received a total dose of 346mg while the rats receiving the modified hemoglobin received a lower total dose of 234mg. These doses were used because the unmodified SFH was excreted in the urine to a greater extent than the cross-linked hemoglobin. Preliminary experiments were done to determine the doses of the two forms of SFH that resulted in comparable blood levels of plasma free hemoglobin during the experimental period. The characteristics of the two SFH solutions are provided in Table 2.

Analytical Methods

Concentrations of methoxy-{3H} inulin in urine and plasma were determined by liquid scintillation counting. Urine and plasma concentrations were measured by flame photometry. Inulin clearance (GFR), and fractional sodium excretion (FeNa) were calculated using standard formulae.

A blood gas analyser (Instrumentation Laboratory, Model 1312, (Lexingon, MA.) was used to measure the p02, pCO2 and pH of the SFH solutions (Table 2). Total free plasma and urine hemoglobin was measured as previously described (12). Oxy- and methemoglobin were measured with a co-oximeter (Instrumentation Laboratory, Model 282, Lexingon, MA.). The p50 of the hemoglobin preparations was measured with a Hemoxanalyser (2). Oncotic

pressure of the SFH solutions was measured using a Wescor Oncometer (Model 4400)

Statistics

The results obtained for each variable during each experimental clearance period were meaned. All data are presented as the means ± SEM. All comparisons within each group (between experimental periods) were made using analysis of variance (ANOVA) followed by the Scheffe test. Comparisons between groups during the same experimental periods were made by unpaired student t-test using the Bonferroni correction where appropriate. Statistics were calculated on a Macintosh computer using the Statworks® software program. A p value of <0.05 was considered significant.

Abbreviations.

SFH= stroma free hemoglobin

MAP=mean arterial pressure

GFR=glomerular filtration rate

FeNa=fractional excretion of sodium

L-NAME= Nω–nitro-L-arginine-methyl ester

RESULTS

1. Effects of modified and unmodified SFH on MAP and GFR and sodium excretion.

Hemorrhage resulted in a comparable fall in blood pressure as well as GFR in all groups. The administration of both the unmodified and the modified (o-raffinose polyhemoglobin) SFH solutions to the hypotensive rats resulted in a rise in MAP as well as GFR to levels comparable to baseline. The two albumin solutions, matched oncotically to each SFH solution (Table 2), also increased MAP and GFR. However, neither albumin control solution increased these values to the same extent as the SFH solutions; in both control albumin groups the MAP

and GFR remained substantially below baseline levels after the albumin solutions were infused (Tables 3 and 4).

The MAP and GFR values reached post-infusion with both unmodified and modified solutions of SFH, were higher than with the albumin control solutions (Tables 3 and 4; Figures 1 and 2).

Hemorrhage was associated with a fall in urine flow rate in all groups and subsequent infusion of albumin control solutions and SFH solutions increased urine flow rates to levels comparable to baseline (Table 3 and 4). FeNa did not change in response to hemorrhage or infusion of albumin or SFH in any of the three groups studied (Tables 3 and 4). However, the unmodified SFH resulted in a substantial diuresis and natriuresis. (Table 3; Figure 3).

2. Effect of nitric oxide synthase (NO) inhibition on MAP following unmodified SFH infusion.

Hemorrhage resulted in an equivalent fall in MAP in all three groups (vehicle, albumin and unmodified SFH (Table 5)). While infusion of the vehicle after hemorrhage had no effect on MAP, both albumin and unmodified SFH increased MAP when administered after hemorrhage. However, SFH increased MAP to a level comparable to the baseline period (an increase of 80% over post-hemorrhage level) while the increase in MAP with albumin was less marked (30% above post-infusion level) ($p<0.05$) (Table 5, Figure 5).

Subsequent infusion of L-NAME increased MAP in both vehicle and albumin groups to levels comparable to baseline "pre-hemorrhage" values. In marked contrast, L-NAME had no effect on MAP in the SFH infused group (Table 5, Figure 5).

3) Renal handling of unmodified and modified SFH.

The unmodified SFH was rapidly excreted in the urine at a rate of 2.3±0.8 mg/minute while the cross-linked hemoglobin was excreted in very small amounts (0.02 ± 0.002 mg/min)($p<0.05$)(Figure 4). The concentrations of red cell-free plasma hemoglobin measured in blood samples obtained at the end of

the experiment were no different between rats receiving the unmodified SFH (1.7±0.3g%) and those receiving the cross-linked SFH (1.6±0.1g%).

DISCUSSION

The administration of SFH following hypotension induced by hemorrhage represents a relevant model to test the potential benefits and toxic effects of these agents (8). We have compared the short term systemic and renal effects of modified and unmodified forms of SFH with albumin solutions of comparable colloid osmotic pressures (Table 1). These albumin solutions are likely to produce approximately the same degree of intravascular volume expansion as the SFH solutions to which they were oncotically matched.

As expected, both albumin solutions resulted in increases in both blood pressure and GFR when infused into the hypotensive rat (Tables 3 and 4), effects likely due to intravascular volume expansion. The unmodified and modified solutions of SFH increased MAP to values comparable to baseline, pre-hemorrhage values. Both SFH solutions increased MAP to a substantially greater extent than the oncotically matched albumin solutions (Tables 3 and 4, Figure 1). Thus both SFH solutions had a hypertensive effect that was independent of the colloid activity of these solutions.

There is substantial evidence that hemoglobin increases systemic blood pressure in normotensive animals (1) and in humans (25). Recently, hemoglobin has also been reported to cause a marked increase in blood pressure in swine following hemorrhagic hypotension (8), a result comparable to that reported in this study. The hypertension induced by hemoglobin has been demonstrated to be due to systemic vasoconstriction (8).

Hemoglobin binds and inactivates NO, converting it to nitrate (10,22) and inhibits NO mediated vasodilation (10,13). This effect of hemoglobin on NO appears to occur only with red cell free preparations of hemoglobin (10). For reasons that remain unclear, hemoglobin within intact red cells does not have any effect on NO mediated vasoactivity (10).

Studies examining the effects of competitive inhibitors of NO production have demonstrated that NO synthase inhibition causes marked vasoconstriction and hypertension in experimental animals (11,14,18). In contrast to NO synthase inhibitors such as L-NAME, hemoglobin reduces NO activity by binding to and inactivating the NO molecule rather than by inhibiting its production (10,22). However, the functional results of the interaction between cell-free hemoglobin and NO is likely to be comparable to that of NO synthase inhibition i.e. reduced availability of NO and consequent systemic vasoconstriction. There is evidence to support the hypothesis that the vasoconstrictor effect of SFH is due, at least in part, to reduced availability of NO (13,16).

In order to determine the extent to which the elevation in MAP induced by SFH in hypotensive rats in this study is due to the effects on NO availability, we examined and compared the effects of NO inhibition with L-NAME on MAP after infusion of control solutions (vehicle and albumin) and unmodified SFH. L-NAME infusion increased MAP to baseline "pre-hemorrhage" values, in hypotensive rats treated with either the vehicle or albumin (Table 5, Figure 5). These data are consistent with our previous report examining the effects of NO inhibition in a comparable model of hemorrhagic hypotension (14). In contrast, SFH infusion alone increased MAP to baseline and resulted in a substantially greater increase in MAP than either vehicle or albumin. Most importantly, L-NAME administration following SFH infusion had no further effect on MAP (Table 5, Figure 5).

The absence of a hypertensive response following NO synthase inhibition in the rat already treated with unmodified SFH is consistent with our hypothesis that the NO system is already maximally inactivated by SFH. Thus it is likely that the hemoglobin-mediated inactivation of constitutively produced NO mediates part of the peripheral vasoconstriction induced by SFH (13,16). We estimate that about 35% of the effect of unmodified SFH on MAP in this study is due to the oncotic effects of albumin while the remaining and predominant elevation in MAP (aproximately 65%) is related to inactivation of NO.

Both modified and unmodified solutions reversed the functional renal failure caused by the low renal perfusion pressure in the hemorrhaged rats. Clearly, this improvement in GFR is the result, at least in part, of increased renal perfusion pressure into the autoregulatory range associated with SFH infusion. However, if we postulate that the hypertensive effect of SFH is mediated by peripheral vasoconstriction due to the inactivation of NO, this response of GFR to SFH is paradoxical, since a number of investigators have clearly demonstrated that inhibition of NO production causes intrarenal as well as non-renal vasoconstriction in animals and decreases both renal plasma flow and GFR in normotensive, euvolemic animals (11,18,20).

However, we have evidence to support the hypothesis that the beneficial effect of SFH solutions on GFR demonstrated in our hypotensive rats can be explained by reduced availability of NO. Our group has reported that NO synthase inhibition produces opposite effects on GFR in normotensive and hypotensive animal (14,15) While NO inhibitors reduce GFR in the normotensive animals (11,18,20), the same intervention markedly improves renal function in rats subjected to hypotensive hemorrhage (14,15).

In a recent report, we have demonstrated that the improvement in GFR induced by NO synthase inhibition in the hypotensive rat was associated with a comparable increase in renal as well as total peripheral resistance (15). The increase in renal resistance in this study was associated with a marked increase in the filtration fraction (15). We concluded from these results that the increase in GFR induced by L-NAME in the hypotensive, hypovolemic rat was due to the combined effects of an increased renal perfusion pressure and preferential intrarenal constriction of the efferent arteriole. Clearly, additional studies are necessary to examine the mechanisms by which GFR is increased by SFH in the hemorrhaged rat, and whether the increase in renal function is asociated with a rise or fall in renal plasma flow. If SFH is inducing intrarenal constriction there is reason to be concerned that higher doses of SFH followed

for a longer period may induce renal ischemic injury by reducing medullary oxygen delivery (4).

We have also demonstrated that while only relatively small amounts of the modified hemoglobin was excreted in the urine, there was substantial urinary loss of the unmodified form of SFH (Figure 4). The rate of unmodified SFH was approximately 100 fold that of the modified SFH. A total of approximately 92 mg of unmodified SFH was excreted during a 40 minute period representing about 25% of the total dose of unmodified hemoglobin (346 mg) that was infused. In contrast, only 0.8mg of modified SFH was excreted in the urine during the same time period, an amount that represents less than 0.5% of the total dose given (234mg). The loss of unmodified SFH in the urine likely explains the observation that while the total dose of unmodified SFH administered was substantially larger than that of modified stroma free hemoglobin, the final concentration of SFH in the plasma measured at the end of the experiment was comparable between the two groups. Thus, the method used to polymerize the modified hemoglobin tested in this study was clearly effective in minimizing urinary excretion of the molecule.

While the modified and unmodified forms of SFH produced comparable effects on MAP and GFR, the effects of these two preparations on urine flow rate and sodium excretion were strikingly different. While neither albumin solutions or the modified, cross-linked form of SFH resulted in any change in either of these variables, the infusion of the unmodified form of hemoglobin was associated with a profound increase in urine flow rate and sodium excretion. (Table 3, Figure 3). Similar effects on sodium and water handling by the kidney have been reported in response to unmodified SFH by other investigators (8). While the profound natriuresis induced by unmodified SFH probably represents a direct toxic effect of the filtered hemoglobin on tubular function, the mechanism responsible for this effect was not determined in this study. Interestingly, hemoglobin has been shown to inhibit Na/K ATPase activity

in neuronal cells (19). Further studies are necessary to elucidate the mechanism/s by which hemoglobin or its metabolites may derange the normal transport function of renal tubular cells.

It is important to emphasize that relatively low plasma concentrations of SFH (1.6-1.7g/100ml) were achieved in this study. Thus it is unlikely that any of the systemic or renal effects of the SFH solutions were due to alterations in the oxygen carrying capacity of the blood. The effects of the far higher concentrations of SFH necessary to alter oxygen carrrying capacity and therefore provide therapeutic benefit need to be examined.

While the hemodynamic effects of the SFH solutions resulted in short term improvements in MAP and GFR in this study, the vasoconstrictive effects of hemoglobin clearly have the potential to cause deleterious effects in more prolonged studies. The systemic vasoconstriction induced by SFH has been shown to prevent the major anticipated benefit of this therapy, i.e. improvement in oxygen delivery to tissues (8). Other studies have shown that SFH induces both pulmonary (8) and coronary vasoconstriction (21), effects that may have harmful effects on pulmonary and/or cardiac function. Also, long term studies are necessary to exclude potential nephrotoxic effects of SFH solutions. The degradation products of hemoglobin that are particularly toxic to renal cells have been shown to accumulate in the kidney days after administration of modified as well as unmodified SFH solutions and may result in delayed nephrotoxicity (5,23). For all these reasons, additional studies are necessary to determine whether any particular form of modified SFH administered in large amounts is beneficial and free of serious side effects or poses risks of unacceptable systemic and renal side effects.

In summary, unmodified SFH is readily filtered at the glomerulus, as expected, and induces a pathological natriuresis. In contrast, the modified SFH is filtered and excreted in extremely small amounts, indicating successful cross-linking of the tetrameric molecule in this particular preparation. Both

modified and unmodified forms of hemoglobin result in marked improvement in blood pressure as well as GFR in hypovolemic and hypotensive rats with functional renal failure. These systemic and renal hemodynamic effects appear to be due to the combined effects of volume expansion and peripheral vasoconstriction resulting from reduced availability of constitutively produced nitric oxide.

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REFERENCES.

1. AMBERSON, W.R., J.J. JENNINGS, and C.M. RHODE. Clinical experience with hemoglobin-saline solutions. J. Appl. Physiol. 1:469-489, 1949.

2. ASAKURA, T., and M.P. REILLY. Methods for the measurement of oxygen equilibration curves of red cell suspensions and hemoglobin solutions, in Oxygen transport in red blood cells. Ed. C. Nicolau, Pergamon Press, 1986, pp 57-75.

3. BRAUN, S., F.R. WEISS, A. KELLER, J.R. CICCONE, and H.G. PREUSS. Evaluation of the Renal Toxicity of Heme Proteins and Their Derivations: A Role in the Genesis of Acute Tubular Necrosis. J Exp Med 129:909-924, 1969.

4. BREZIS, M., S.N. HEYMAN, D. DINOUR, F.H. EPSTEIN, and S ROSEN. Role of nitric oxide in renal medullary oxygenation. J. Clin. Invest. 88:390-395, 1991.

5. BUNN H.F., and J.H. JANDL. Renal Handling of Hemoglobin-II. Catabolism. J Exp Med 129:925934, 1967.

6. CHANG, T.M.S. Modified Hemoglobin in Blood Substitutes and Oxygen Carriers ed. Chang TMS. Marcel Dekker, Inc., New York, 1993, pp 3-23.

7. GUIDET, B., and S.V.SHAH. Enhanced In Vivo H_2O_2 Generation by Rat Kidney by Glycerol-Induced Renal Failure. Am J. Physiol 257:26:F4400-F445, 1989.

8. HESS,J.R., V.W. MACDONALD and W. BRINKLEY. Systemic and pulmonary hypertension after resuscitation with cell-free hemoglobin. J. Appl. Physiol. 74 (4): 1769-1778, 1993.

9. JAENIKE, J.R. The renal lesion associated with hemoglobinemia. J. Exp. Med. 123:523-535,1966.

10. KILBOURN, R.G., J. GHISLAINE, B. CASHON, J. DeANGELO, , AND J. BONAVENTURA. Cell-free hemoglobin reverses the endotoxin mediated hyporesponsivity of rat aortic rings to α-adrenergic agents. Biochem. Biophys. Res. Comm. 199:155-162,1994.
BBRC Feb 1994.

11. LAHERA, V., M.G. SALOM, F. MIRANDA-GUARDIOLA, S. MONCADA, and J.C. ROMERO. Effects of N^G-Nitro-L-Argninine Methyl Ester on Renal Function and Blood Pressure. Am J Physiol 261:30:F1033-F1037, 1991.

12. LIEBERTHAL, W., E.F. WOLF, E.W. MERRILL, N.G. LEVINSKY, and C.R. VALERI. Hemodynamic Effects of Different Preparations of Stroma Free Hemolysates in the Isolated Perfused Rat Kidney. Life Sci 41:2525-2533, 1987.

13. LIEBERTHAL, W., W.M. VOGEL, C.S. APSTEIN, and C.R.VALERI. Studies of the Mechanism of the Vasoconstrictor Activity of Stroma-Free Hemoglobin in the Isolated Perfused Rat Kidney ant Rabbit Heart. Editor: G. Brewer. The Red Cell: Seventh Ann Arbor Conference, pp 407-422, 1989.

14. LIEBERTHAL, W, A.E. McGARRY, J. SHEILS and C.R. VALERI. Nitric Oxide inhibition in Rats Improves Blood Pressure and Renal Function During Hypovolemic Shock. Am J Physiol 261:30:F868-F872, 1991.

15. THOMPSON, A., C.R. VALERI, and W LIEBERTHAL. Nitric oxide inhibition reverses renal failure induced by hemorrhagic hypotension. J. Am. Soc. Nephrology 4:570,1993.

16. MONCADA, S., R.M.J. PALMER, and E.A. HIGGS. Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacol. Rev. 40:109-142, 1991.

17. PALLER, M.S. Hemoglobin-and Myoglobin-Induced Acute Renal Failure in Rats: Role of Irone in Nephrotoxicity. Am J Physiol 255:24:F539-F544, 1988.

18. REES, D.D., R.M.J. PALMER, and S. MONCADA. Role of endothelium derived nitric oxide in the regulation of blood pressure. Proc. Natl. Acad. Sci. USA 86:3375-3378, 1989.

19. SADRADEH, S.M.H., D.K. ANDERSON, H.E. HALLOWAY, and J.E. EATON. Hemoglobin potentiates central nervous system damage. J. Clin. Invest. 79:662-664, 1987.

20. TOLINS, J.P., R.M.J. PALMER, S. MONCADA AND L. RAIJ. Role of endothelium derived relaxing factor in regulation of renal hemodynamic responses. Am. J. Physiol. 258 (Heart Circ. Physiol. 27): H655-H662, 1990.

21. VOGEL, W.M., R.C. DENNIS, G. CASSIDY, C.S. APSTEIN, and C.R. VALERI. Coronary Constrictor Effect of Stroma-Free Hemoglobin Solutions. Am J Physiol 251: (HEART CIRC. PHYSIOL. 20):H413-H420, 1986.

22. WENNMALM, A., BENTHIN, G., AND PETERSSON, A.S. Dependence of the metabolism of mitric oxide by human whole blood on the oxygenation of its red cell hemoglobin. Br. J. Pharmacol;106:507-508, 1992.

23. WINSLOW, R.M. Metabolism of Hemoglobin in Hemoglobin-Based Red Cell Substitutes. ed R.M. Winslow , Johns Hopkins University Press, Baltimore, London pp 118-153, 1993.

24. WINSLOW R.M. Clinical trials in Hemoglobin-Based Red Cell Substitutes. ed. R.M. Winslow, Johns Hopkins University Press, 1993, pp 136-163.

25. WINSLOW R.M. The Toxicity of Hemoglobin in Hemoglobin-Based Red Cell Substitutes. ed. R.M. Winslow, Johns Hopkins University Press, 1993, pp 175-184.

26. ZAGER, R.A., and L.M. GAMELIN. Pathogenetic Mechanisms in Experimental Hemoglobinuric Acute Renal Failure. Am J Physiol 256:25 F446-F455, 1989.

LEGENDS

Figure 1

Effects of unmodified SFH and modified SFH on mean arterial pressure (MAP) following hemorrhage compared to the effects of two different control albumin solutions matched oncotically to each SFH solution.

The oncotic pressure of the unmodified SFH was 92.4mmHg and its albumin control was 94.0mmHg. The oncotic pressure of the cross-linked, modified SFH and its control albumin solution was 21.0mmHg (Table 1)

*=$p<0.05$ compared to the albumin solution matched to each SFH solution. n=8 for both control albumin solution groups; n=8 in unmodified SFH group and n=7 in modified SFH group.

Figure 2

Effects of unmodified SFH and modified SFH on glomerular filtration rate (GFR) following hemorrhage compared to the effects of two different albumin solutions matched oncotically to each SFH solution .

*=$p<0.05$ compared to the albumin solution matched to each SFH solution.

Figure 3

Effects of the unmodified SFH and modified SFH on fractional sodium excretion following hemorrhage compared to the effects of two different albumin solutions matched oncotically to each SFH solution (see Table 1).

*=$p<0.05$ compared to the albumin solution matched to each SFH solution.

Figure 4

Urinary excretion rate of unmodified and modified forms of SFH

*p=<0.05 compared to unmodified SFH.

Figure 5

Effect of nitric oxide synthase inhibition with L-NAME following infusion of the vehicle (speckled bar), albumin solution (cross-hatched bar) and unmodified SFH (black bar)

*=$p<0.05$ compared to same group (albumin or SFH) during post hemorrhage period

†=$p<0.05$ compared to same group (vehicle or albumin) during post infusion period

¶=$p<0.05$ compared to other groups within the same period (post-infusion of vehicle, albumin or unmodified SFH).

n=4 in all groups.

Table 1

Comparison of concentrations and oncotic pressures of the unmodified and modified forms of SFH and the control albumin solutions.

	Concentration (g/dL)	Oncotic pressure (mmHg)
Unmodified SFH solution	17.3	92.4
Control albumin solution for unmodified SFH	16.8	94.0
Modified SFH solution	11.7	21.0
Control albumin solution for modified SFH	5.0	21.0

Table 2
Characteristics of the unmodified and modified (o-raffinose cross-linked) stroma free hemoglobin (SFH) solutions

	Unmodified SFH	Cross-linked SFH
O_2 Hemoglobin (%):	96.7	86.6
Co Hemoglobin (%):	3.5	2.5
Methemoglobin (%):	0.2	6.6
Volume O_2 (%):	23.2	14.0
p50 (Torr at pH 7.4):	14.0	22.2

Table 3

Effects of unmodified SFH compared to an oncotically matched albumin solution on mean arterial pressure (MAP), glomerular filtration rate (GFR) urinary flow rate and fractional sodium excretion (FeNa) following hemorrhage

	BASELINE PERIOD	POST-HEMORRHAGE PERIOD	POST-INFUSION PERIOD
MAP (mmHg)			
Albumin group	114 ± 2	63 ± 3*	82 ± 2*†¶
Unmodified SFH	110 ± 5	61 ± 6*	111 ± 7†
GFR (ml/min)			
Albumin group	3.0 ± 0.2	1.0 ± 0.2*	2.0 ± 0.2*†¶
Unmodified SFH	2.8 ± 0.3	1.1 ± 0.5*	2.7 ± 0.3†
Urine flow rate (ul/min)			
Albumin group	7.8 ± 0.6	4.1 ± 0.9*	8.5 ± 0.9†¶
Unmodified SFH	8.3 ± 1.0	4.0 ± 0.9*	8.1 ± 1.1†
FeNa (%)			
Albumin group	0.08 ± 0.02	0.08 ± 0.04	0.06 ± 0.02¶
Unmodified SFH	0.10 ± 0.04	0.06 ± 0.03	2.10 ± 0.60*†

*=$p<0.05$ vs. baseline period within albumin or unmodified SFH groups.
†=$p<0.05$ vs. post-hemorrhage period within albumin or unmodified SFH groups.
¶=$p<0.05$ vs unmodified SFH group within post-infusion period.

Table 4

Effects of SFH modified by o-raffinose crosslinking compared to an oncotically matched albumin solution on mean arterial pressure (MAP), glomerular filtration rate (GFR), urine flow rate and fractional sodium excretion (FeNa) following hemorrhage

	BASELINE PERIOD	POST-HEMORRHAGE PERIOD	POST-INFUSION PERIOD
MAP (mmHg)			
Albumin group	109 ± 2	59 ± 4*	71 ± 5*†¶
Modified SFH	105 ± 4	56 ± 4*	96 ± 5†
GFR (ml/min)			
Albumin group	3.1 ± 0.3	1.0 ± 0.3*	1.9 ± 0.2*†¶
Modified SFH	2.5 ± 0.2	0.7 ± 0.2*	2.7 ± 0.3†
Urine flow rate (ul/min)			
Albumin group	13.5 ± 0.6	2.5 ± 0.5*	11 ± 1.9†
Modified SFH	7.8 ± 1.0	4.0 ± 1.4*	9.4 ± 3.6†
FeNa (%)			
Albumin group	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.02
Modified SFH	0.07 ± 0.02	0.06 ± 0.02	0.08 ± 0.04

*=$p<0.05$ vs baseline period within albumin or modified SFH groups
†=$p<0.05$ vs post-hemorrhage period within albumin or modified SFH groups
¶=$p<0.05$ vs modified SFH group within post-infusion period.

Table 5

Effect of nitric oxide inhibition with L-NAME on mean arterial pressure (MAP)(mmHg) after administration of the vehicle, oncotically matched albumin or unmodified SFH following hemorrhagic hypotension

	BASELINE PERIOD	POST-HEMORRHAGE PERIOD	POST-INFUSION PERIOD	L-NAME PERIOD
Vehicle group	113 ± 3	60 ± 3*	61 ± 2*§	94 ± 8*†¶
Albumin group	108 ± 3	63 ± 3*	82 ± 4*†§	104 ± 7†¶
SFH group	108 ± 2	66 ± 1*	119 ± 3†§	112 ± 3†

*=p<0.05 vs baseline period within each group
†=p<0.05 vs post-hemorrhage period within each group
¶=p≤0.05 vs post-infusion period within each group
§=p<0.05 vs. other groups within post-infusion period

Figure 1

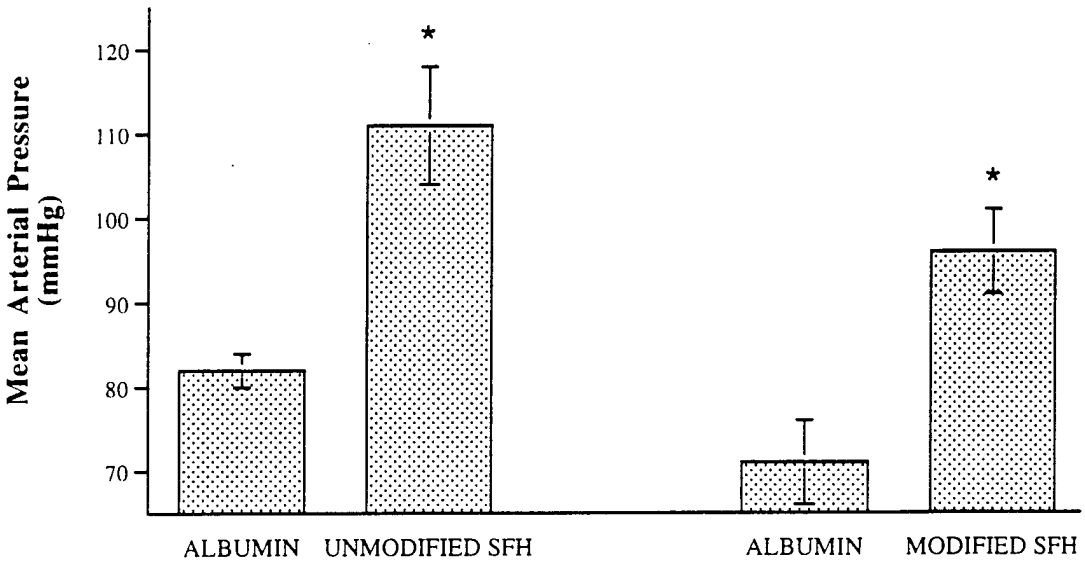

Mean Arterial Pressure (mmHg)
120
110
100
90
80
70
*
*
ALBUMIN
UNMODIFIED SFH
ALBUMIN
MODIFIED SFH

Figure 2

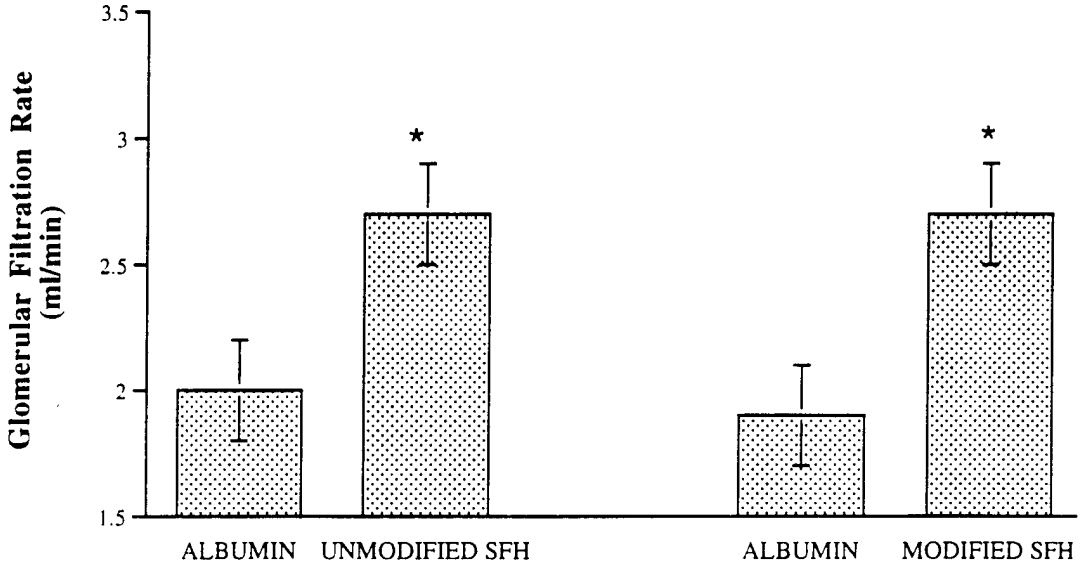

Glomerular Filtration Rate (ml/min)
3.5
3
2.5
2
1.5
*
*
ALBUMIN
UNMODIFIED SFH
ALBUMIN
MODIFIED SFH

Figure 3

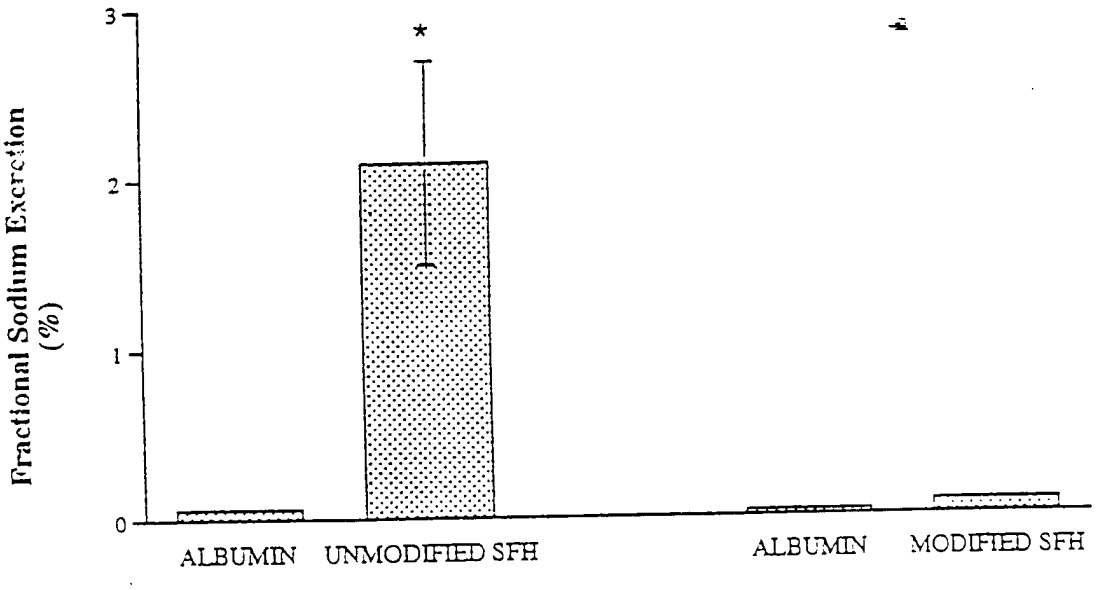

Fractional Sodium Excretion (%)
3
2
1
0
*
ALBUMIN
UNMODIFIED SFH
ALBUMIN
MODIFIED SFH

Figure 4

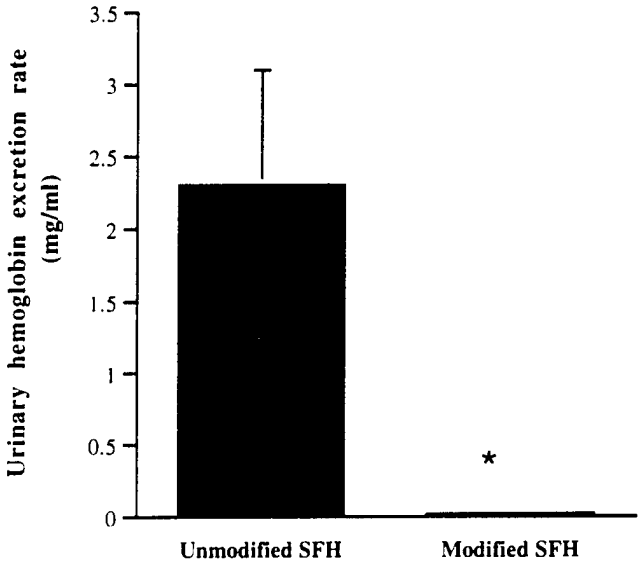
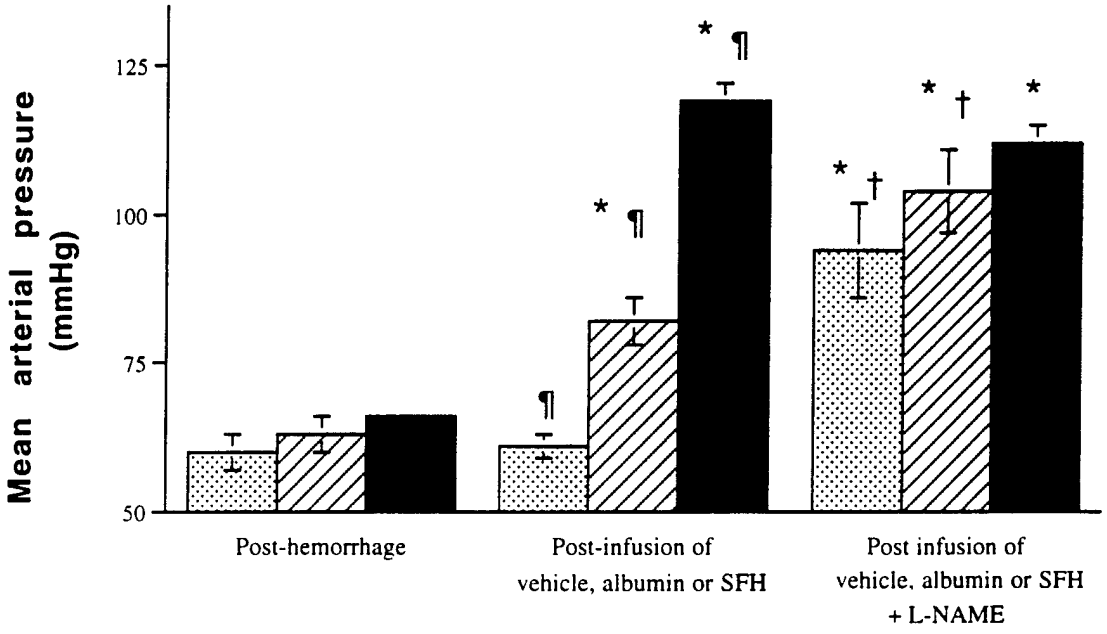

Urinary hemoglobin excretion rate (mg/ml)
3.5
3
2.5
2
1.5
1
0.5
0
*
Unmodified SFH
Modified SFH

Figure 5


Mean arterial pressure (mmHg)
125
100
75
50
*
¶
†
Post-hemorrhage
Post-infusion of vehicle, albumin or SFH
Post infusion of vehicle, albumin or SFH + L-NAME